# Preservative and irritant capacity of biosurfactants from different sources: a comparative study

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**ABSTRACT**

One of the most important challenges for pharmaceutical and cosmetic industries is the solubilization and preservation of their active ingredients. Therefore, most of these formulations contain irritant chemical additives in order to improve their shelf-life and the solubility of hydrophobic ingredients. An interesting alternative to chemical surfactants and preservatives is the use of biosurfactants, thus their surfactant properties and composition make them more biocompatible than their chemical counterparts. Moreover, some biosurfactants have shown antimicrobial activity, in addition to their detergent capacity.

In this work, it was studied the antimicrobial and irritant effect of two biosurfactant extracts, one produced in a controlled fermentation with *Lactobacillus pentosus* and other produced from corn stream by spontaneous fermentation.

The results showed a strong antimicrobial activity of the biosurfactant extract obtained from corn stream on pathogenic bacteria, in comparison with the *L. pentosus* biosurfactant extract. Moreover, both biosurfactants did not produce any irritant effect on chorioallantoic membrane of hen´s egg assay contrarily to sodium dodecyl sulfate. This is the first study dealing with the application of biosurfactant extracts on sensitive biological membranes and the first time that it is evaluated the preservative capacity of a biosurfactant extract obtained in spontaneous fermentation, achieving promising results.

**Keywords**: *biosurfactant; preservative; non-irritant; corn; Lactobacillus pentosus; Escherichia coli; Candida albicans; Aspergillus brasiliensis.*

## INTRODUCTION

An important aspect in different industrial sectors like the cosmetic and pharmaceutical industries is the preservation and solubilization of active ingredients in their formulations. For that, surfactants and/or preservatives are usually chosen. Most of them comprise chemical compounds, which in some cases can produce irritant responses [1]. Over the past few years, the demand of cosmetic and pharmaceutical industries for products obtained from natural sources has significantly increased. In this sense, it is being looked for more biocompatible preservatives, with lower harmful effects than their chemical counterparts [2–7].

Regarding pharmaceutical and cosmetic formulations, the preservative, irritant and emulsifying properties of biosurfactants are the most relevant. However, the solubilization and dispersion of certain kind of solutes is also something to take into consideration, thus more than 40% of the new active ingredients discovered in pharmaceutical industry are practically insoluble in water [8].

Poorly water-soluble ingredients often require high doses of surfactants in order to reach therapeutic plasma concentrations after oral administration. Furthermore, various techniques consisting of physical and chemical modifications of drugs, as well as other methods like particle size reduction, crystal engineering, among others, are used for the enhancement of the solubility of badly soluble substances in water. However, the use of surfactants is a basic and oldest methodology[8], still used in many cosmetic and pharmaceutical formulations.

Recently, it has been demonstrated that biosurfactants are able to improve the preservation and solubility of active ingredients commonly used in these formulations, being a good alternative to synthetic ones, which can cause important damages [9–11].

Moreover, it has been also proved that some biosurfactants, used as solubilizing agents, possess antimicrobial activity [12–17].

In comparison with chemical surfactants, biosurfactants should be more biodegradable because they are composed by lipids, proteins or peptides, and/or carbohydrates [18,19]. Besides, it has also been demonstrated that they can produce a synergetic effect on some antibiotics, improving their antimicrobial activity and their solubilization [20], this is not strange because it has been demonstrated that many biosurfactants has antimicrobial properties [13,14,21].

The aim of this work is to study the antimicrobial and irritant properties of a natural biosurfactant extract, obtained from the spontaneous fermentation of an aqueous stream of corn milling industry and a biosurfactant, obtained from *L. pentosus* in a controlled fermentation. In comparison with other biosurfactants these have the advantage that are produced in presence of lactic acid bacteria.

## MATERIALS AND METHODS

### *Production of biosurfactant extracts*

*Biosurfactant from corn stream (BS-CSL)*

Corn steep liquor (CSL) is a corn stream, coming from the corn wet-milling industry, spontaneously fermented by lactic acid bacteria, which are "Generally Recognized As Safe" (GRAS) by the US Food and Drug Administration (FDA). It is important to underline that the biosurfactant extract obtained from CSL was evaluated in previous works, observing its prebiotic effect on *Lactobacillus casei,* that confirms its non-toxicity.[22]

For the biosurfactant production, CSL provided by Santa Cruz Biotechnology (Lot L1813), with a solid content of 50%, was used. Previously to the extraction, CSL was

diluted in demineralized water up to 50 g/L. The biosurfactant extract was obtained by liquid-liquid extraction, by triplicate, following the protocol established by Vecino and collaborators in previous work [23]. After extraction, the biosurfactant was separated from the organic phase by rotary evaporation, and dissolved in demineralized water up to 1 g/L. The biosurfactant extract was filtered through 0.22 μm PVDF membrane (Millipore, USA).

*Biosurfactant from Lactobacillus pentosus (BS-pentosus)*

*L. pentosus* CECT-4023T (ATCC-8041) was obtained from the Spanish Type Culture collection (CECT) (Valencia, Spain). This strain was grown for 24 h in a 250 mL Erlenmeyer flask, with 100 mL of complete medium, so-named by its inventors (de Man, Rogosa and Sharpe), MRS Broth, at 31 ºC and 200 rpm. This was used as inoculum for a 1.5 L fermentation, in which the culture medium contained 11 g/L of glucose and 18 g/L of xylose. Moreover, the medium was also supplemented with 10 g/L of CSL and 10 g/L of yeast extract as nitrogen source. Finally, it was sterilized (121 ºC for 15 min) and the inoculum added, and grown for 48 h at 31 ºC, pH of 5.85 and 200 rpm, in an Applikon fermenter.

Afterwards, the fermentation medium was centrifuged, and the biomass was washed with distilled water twice. Then, biomass was re-suspended in 250 mL of phosphate buffer saline (PBS) (10mM $KH_2PO_4/K_2HPO_4$ with 150 mM NaCl). The extract obtained was dialyzed against demineralized water at 4 ºC in a Spectra/Por ® dialysis membrane (molecular weight cut-off 6000-8000 Da; Spectrum Laboratories, Inc., USA) for 48 h, and, finally, the solution was lyophilized using a LyoQuest HT40 (Telstar, USA), obtaining a white powder. The solution used during this experiments was prepared dissolving this powder in distilled water up to 1 g/L, for comparative purposes.

***Characterization of biosurfactant extracts***

*Critical micellar concentration*

When the concentration of biosurfactant is under its critical micellar concentration (CMC), a linear relationship between the surface tension of the solution and the log of concentration of a specific biosurfactant can be established [12,24]. Thus, to determine the CMC of both biosurfactant extracts, different concentrations were prepared. Finally, the surface tension of each sample was measured using a tensiometer KRÜSS (K20 EasyDyne) provided with a Wilhelmy plate method (see **Figures 1** and **2** in the Supplementary Information). Measurements were made by triplicate to increase the accuracy of the results. The surface tension value of demineralized water, 72 mN/m, was used as negative control.

*Biochemical analysis*

Biosurfactants are usually formed by biomolecules such as proteins, lipids and/or sugars [25]. For the lipids determination, it was used Sulfo-phophovanillin (SPV) method, which allowed to extract and quantify the total amount of lipids [26]. For that, 5 mL of a chloroform/methanol solution (1:1 v/v) was used to dissolve 100 mg of biosurfactant extract. Then, it was mixed and kept at 4 ºC overnight. Secondly, 1 mL of 0.9% NaCl was added, and the phase which contained lipids (chloroform) was collected. Once the lipid extract was obtained, 80 µL of sample solution were dissolved in 720 µL of concentrated $H_2SO_4$ (Panreac, Spain), heated at 100 ºC for 10 min, and final cooled to room temperature. Then, 2mL of vanillin-phosphoric acid reagent was added and heated again at 37 ºC for 15 min for color developing. The sample was cooled again, and storage for 45 min in a dark box, and measured with a Horiba OCMA-130 oil content analyzer. Cholesterol was used as standard for calibration.

Additionally, total organic C, O, N and H were determined using thermal conductivity detection (TCD). The protocol consists of the sample decomposition by combustion,

and its analysis using thermal conductivity detection (TCD) on a Carlo Erba EA-1108CHNS-O element analyzer [27]. The % of N was converted in peptide content by multiplying by 6.25 [28].

*Mass spectrophotometry analysis of biosurfactant*

Electrospray ionization mass spectrometry/collisin-induced disociation (ESI-MS/CID) was used to characterize all the compounds present in both extracts studied. For that, 1 mg of the sample was diluted in chloroform, and volatilized under vacuum. Then, a current of electrons was used for ionize the molecules, and the fragmentation pattern was recorded ESI-MS/CID spectra were recorded on a Mass Spectrometer Bruker FTMS APEXIII (Bruker, USA) in positive mode.

***Microbiological quality test***

Before studying the antimicrobial capacity of both biosurfactant extracts, this was subjected to a microbiological quality test, in order to confirm the absence of microorganisms, in the biosurfactant extracts. In **Table 1**, it was included the microorganisms evaluated, all of them provided by the Spanish Type Culture Collection (CECT, Spain), as well as their growth conditions. It was tested the presence of any harmless microorganism (e.g. genus *Lactobacillus*), and the pathogenic ones included in the Challenge Test. For that, 49.54 mL of Eugon LT 100 broth medium was mixed with 5.15 g of the aqueous solution of biosurfactant extract for 20 min, in order to neutralize the possible preservative effect of the biosurfactant. Afterwards, the samples were grown in Blood Agar for detecting bacteria, and Sabourad Dextrose Agar, in case of yeasts and fungi. Selective growth media as Cetrimide, Sabouraud Dextrose Cloranfenicol Agar, Baird Parker, and Mc Conkey, for *P. aeruginosa*, *Candida albicans*, *E. coli*, and *S. aureus* and *Aspergillus brasiliensis*, respectively, were used. Samples were incubated between 48-72 h at 32 ºC (see **Table 1**).

***Study of the antimicrobial and preservative capacity of biosurfactant extracts***

The antimicrobial activity of the biosurfactant extracts were analyzed following the ISO 11930:2012 procedure, named Challenge test [29]. This protocol is based on the inoculation of a product or a solution with a known inoculum of 5 relevant strains of microorganisms: *E. coli*, *S. aureus*, *C. albicans*, *P. aeruginosa* and *A. brasiliensis*.

20 mL of the aqueous solution of biosurfactant extract at 1 g/L was inoculated with each microorganism in 5 different test tubes, achieving a concentration about $10^6$ CFU/mL for bacteria, and around $10^4$ CFU/mL for yeasts and fungi. Then, 1 mL of these mixtures was removed after 7, 14 and 28 days, and neutralized using 9 mL of Eugon LT 100 broth medium. Immediately, the samples were incubated 3 days in Tryptic Soy Agar at 32 ºC for bacterias, and 5 days in Sabouraud Dextrose Cloranfenicol Agar at 22 ºC, for yeasts and fungi. Finally, the quantification of microorganisms was carried out, determining the new concentration in CFU/mL (see **Table 1**).

Following the log reduction achieved (L) is calculated and converted into percentage of microbial load reduction (R) following the **Equation 1** and **Equation 2**, respectively.

$$L = [\log_{10}(A) - \log_{10}(B)] \qquad Eq.1$$

$$R\ (\%) = [1 - 10^{-L}]\ x\ 100 \qquad Eq.2$$

where A is the CFU/mL in the basal sample (as control sample) and B is the CFU/mL in the samples treated with biosurfactant extract at different intervals of time.

***Irritant test of the biosurfactant extracts: Hen´s egg test on the chorioallantoic membrane***

White leghorn chicken eggs were incubated for 9 days with automatic rotation at 37.5 ºC. After this time, any defective eggs were discarded. For the assay, the shell around the air cell was removed and the inner membranes were extracted to reveal the

chorioallantoic membrane (CAM) of the hen´s egg. Then, 0.3 µL of the analyzed substances were added to this membrane. NaOH 0.1 N and phosphate buffered saline (PBS) were used as positive and negative control, respectively; whereas biosurfactant extracts were assayed at a concentration of 1.0 g/L, dissolved in PBS. Also, as positive control, a synthetic surfactant was used. In this case, it was chosen sodium dodecyl sulphate (SDS) solution, at the same concentration of the biosurfactant extracts, dissolved in PBS, as well. The changes in the CAM were observed over a period of 5 min, and the time taken for injury to occur was recorded. All the assays were carried out by triplicate.

Irritancy was scored according to the severity and speed of damages, following the recommended protocol by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) [30]. The endpoints observed were as follows: hemorrhage (bleeding from the vessels), vascular lysis (blood vessel disintegration) and coagulation (intra- and extra-vascular protein denaturation). The irritation index (II) was calculated using **Equation 3.**

$$II = [(301 - H) \times 5/300] + [(301 - L) \times 7/300] + [(301 - C) \times 9/300] \qquad Eq.3$$

where H is the time (s) needed to notice presence of hemorrhage, whereas L and C are the time (s) that lysis and coagulation phenomena start to appear, respectively.

## RESULTS AND DISCUSSION

### *Characterization of the biosurfactants extracts under evaluation*

**Table 2** shows the physical and chemical properties of the biosurfactant extracts under evaluation. It can be observed that their properties differ from one biosurfactant to

another. At the maximal concentration of biosurfactant extracted from CSL assayed (1 g/L), it was obtained a surface tension reduction of 32 units. This reduction is similar to the surface tension reduction obtained theoretically at the CMC. In the case of the biosurfactant extract produced by *L. pentosus,* at the maximal concentration assayed (10 g/L), it produced a surface tension reduction of water of 22 units, which is similar to the reduction achieved at the CMC calculated theoretically. It was observed a higher surfactant capacity for the biosurfactant extract from CSL in comparison to the biosurfactant extract produced by *L. pentosus.* Furthermore, the values of CMC also showed important differences, being 0.19 ± 0.05 g/L and 2.16 ± 0.29 g/L for BS from CSL and *L. pentosus*, respectively. These results are in concordance with previous works [14], where it was observed that biosurfactants produced by lactic bacteria, in controlled fermentations, gave CMC values equal or higher than 1.5 g/L [15,16]. Moreover, the CMC determined for the biosurfactant extract obtained from corn stream is in concordance with the CMC previous published by Rincón-Fontán et al. [31,32]. It is noticed that the CMC value of biosurfactant from CSL was lightly higher than other biosurfactants produced by microorganisms such as *Bacillus subtilis, Rhodococcus erythropolis* or *Bacillus flexus*, that gave CMC values of 0.02 g/L, 0.03 g/L and 0.05 g/L, respectively [33–35]. However, Ohadi and collaborators [36] have characterized a lipopeptide produced by *Acinetobacter junii B6*, being its CMC 0.3 g/L. The differences between both biosurfactants under evaluation can be explained in terms of their composition, the biosurfactant produced from *L. pentosus* is a glycolipopeptide, as the chemical analysis revealed the presence of lipids, sugars and organic nitrogen [37] whereas the biosurfactant produced from CSL gave similar properties than lipopeptides biosurfactants [23,38]. This fact can be explained because they are obtained from different sources and under different operational conditions. The biosurfactant obtained from

CSL is produced under extreme conditions during steeping process of corn, which could activate the production of secondary metabolites as biosurfactants [39,40]. On the other hand biosurfactant from *L. pentosus* is obtained under optimum operational conditions. Additionally the microbial biomass involved in both processes is different.

In general, the CMC represents an important parameter for the industrial applications of biosurfactants, because it defines the minimum amount of compound necessary to reach the maximum reduction in the surface tension. Moreover, it represents the concentration of biosurfactant needed to start forming micelles. At the CMC, micelle formation occurs and surfactants entrap the drugs or active ingredients within the micelles, allowing their solubilization. Therefore, in order to be effective, surfactants have to be above their CMC, which is in the range of 0.05–0.10% for most surfactants [8]. However, at these concentrations, most of synthetic surfactants are irritating [7,41].

In terms of their composition, both biosurfactants were composed by fatty acids but in different proportion, thus following the SPV method, it was detected a lipid content of 41.3% for BS from CSL whereas it was only detected a 2.5% in the case of BS from *L. pentosus*. These results are in concordance with those previously published, in which the major fatty acids identified in these biosurfactant extracts were palmitic acid, oleic acid, stearic acid, linoleic acid and linoelaidic acid [21,27,32].

Concerning to ESI analysis, **Figure 1** conveys a comparison between both biosurfactants studied in this work. In the case of BS from CSL (**Figure 1A**), six significant signals were detected, at a m/z of 337, 445, 663, 879, and 999. In this regard, it is common to find ESI main biomarkers for lipopeptide biosurfactants from 966 to 1108 m/z, despite the fact that these biosurfactants present other peaks with lower masses between 400 and 800 Da [42]. For this reason, it can be speculated that the kind of biosurfactant extract obtained from CSL correspond to a lipopeptide. Moreover, the

signals recorded were similar to the peaks identified by other authors as lipopeptides, like fengycin and its homologous [42]. For instance, Bie et al., [43] produced fengycin using a *Bacillus subtilis* strain through a controlled fermentation. Then, fengycin was purified by acid precipitation with HCl 6N followed by extraction with methanol. Additionally, they used a reverse phase C18 analytical column obtaining various extracts. Following, they analysed the extracts by ESI-MS/CID, observing high intense signals at 1449, 765, 773 among other minor signals, depending on the extract. The peaks with lower masses are similar to those obtained for biosurfactant from CSL. This fact is remarkable as fengycin extracts were submitted to different purification processes, observing how the lower m/z signals remained in the final spectra. On the other hand, Li et al., [44] reported high intense signals at 933, 805, 533, 412 m/z in the biosurfactant extract produced by *Bacillus pseudomycoides,* using soybean oil waste as the sole source of carbon and energy. This extract was obtained from cell-free culture supernatant, and was further purified by column chromatography on reverse-phase silica gel, ultra-filtration, acidification and lyophilisation. Finally, it was analysed by GC-MS, amino acid analyser and LTQ Orbitrap Elite combination mass spectrometer. Then, it was observed that the extract contained a novel cyclic lipopeptide with a long-chain fatty acid 3-OH-C18 and a peptide chain of five amino acids. As it was reflected in **Table 3,** the ESI signals for biosurfactant from CSL were in agreement with those obtained by Li et al. [44], presenting peaks with the same m/z., despite the different purification steps.

On the other hand, the biosurfactant extract produced by *L. pentosus* (**Figure 1B**) presented five relevant signals: 245, 369, 473, 685, 782 m/z. Although in the literature there are some publications dealing with glycolipopeptides and glycoproteins [14,21,37] the existence of mass analysis is scarce. For this reason, the signals obtained for biosurfactant extract from *L. pentosus* is compared to those of rhamnolipids and

sophorolipids, thus they are in the same range. Therefore, **Table 3** includes ESI spectra from rhamnolipids, and sophorolipids, with m/z signals at of 677 and 702 [45,46].

According to the elemental analysis, the amount of C was much higher in the BS from CSL, due to the percentage of fatty acids were much higher as well. In the case of BS from *L. pentosus* was remarkable the amount of nitrogen. This value allowed determining the protein content, which represents a 27.5% in comparison to the 4.2% in the case of BS from CSL. That information confirms that biosurfactants are composed by compatible molecules like lipids, proteins or peptides, and/or carbohydrates, contrary to synthetic surfactants. For this reason, their use in cosmetic formulations would avoid side effects of chemical surfactants. Furthermore, the presence of lipids in biosurfactant extracts could also be interesting for improving the skin hydration, or avoiding dryness or itchiness [47–49]. Moreover, the lipid matrix in the stratum corneum plays an important role in the barrier function of the skin [50]. The main lipid classes in this lipid matrix are ceramides, cholesterol and free fatty acids [51], therefore the biosurfactant extracted from CSL could provide natural fatty acids to the skin stratum corneum. On the other hand, the biosurfactant produced by *L. pentosus,* due to its higher concentration in proteins, would be more suitable for hair conditioners or for regenerating the skin [52]. Moreover, the general capacity of biosurfactants to solubilize different oils is an interesting property for their inclusion in oil-based emulsions or creams, confirmed in the case of other biosurfactant produce by *Lactobacillus paracasei* [53]. This fact allows to speculated that both biosurfactant extracts studied in this work, produced in presence of *Lactobacilli* strains could have great potential to solubilize essential oils, as well.

### *Antimicrobial activity of the biosurfactant extracts*

When biosurfactant extracts were growth on generic culture media such as Blood Agar or Sabouraud Dextrose Agar (SDA), no bacteria, yeast of fungi was detected, thus it can

be speculated that this extract did not contain the pathogenic microorganisms under evaluation. The results obtained in the Challenge test for both biosurfactant extracts are shown in **Table 4**. The biosurfactant extract from CSL was able to eliminate *S. aureus* and *E. coli* just in 7 days, while *P. aeruginosa* after 14 days of incubation. Regarding the bactericidal effect observed against these microorganisms, the biosurfactant extract could be catalogued as preservative type A according to the normative ISO 11930:2012 [29]. A cosmetic product can be considered as preservative type A if it reduces in 1 or 3 logarithmic units the count of *C. albicans* or bacteria, respectively, after 7 days of incubation, and it should not be observed any increment in their growth the following days of the assay. On the other hand, a preservative can be catalogued as B if the 1 or 3 logarithmic unit's reduction is achieved after 14 days of incubation for *C. albicans* or bacteria, respectively. Taking into account the growth of *A. brasiliensis*, a product can be considered preservative type A or B when it does not exist any increment in the number of colonies during the first 14 days after inoculation. Moreover, the growth cannot be increased nor reduced in more than 1 logarithmic unit during the next days that lasts the assay.

In comparison, biosurfactant produced by *L. pentosus* did not diminish the bacteria concentration. In fact, the negative value of L (Log reduction) obtained implies an increasing in microorganism colonies, what makes this biosurfactant extract an unsuitable preservative against bacteria.

Regarding to fungi and yeast, the biosurfactant extract from CSL, the preservative effect was lower than that observed on bacteria. In fact, highest reduction of *A. brasiliensis* conidia was achieved after 14 days (L=1.30), diminishing this value after 28 days of conidial load (L=0.70). In regard to *C. albicans*, a qualitative population reduction was observed after 28 days, in terms of log reductions, being the concentration at this time

higher than the basal concentration. A similar behavior was observed in the case of biosurfactant of *L. pentosus*, thus the highest reduction of *A. brasiliensis* was achieved after 14 days (L=0.91), decreasing this value after 28 days as well (L=-0.13). For *C. albicans,* the presence of biosurfactant had increased the yeast conidia.

Finally, the preservative effect of both biosurfactant extracts was also calculated in terms of microbial reduction percentage (R), which was higher than 99.999% for all the bacteria in presence of the biosurfactant extract from CSL, corresponding with at least 6 units of log reduction; whereas in the case of *A. brasiliensis* maximal reduction percentages values, 94.998%, were achieved after 14 days, although after 28 days this reduction decreased up to 80%. Based on this reduction percentage, it can be speculated that the biosurfactant extract obtained from corn, at concentration of 1 g/L, has some antimicrobial activity on *A. brasiliensis* conidia. However, it is not enough to catalogue the biosurfactant extract as preservative against moulds. Regarding *C. albicans*, the biosurfactant extract from CSL promoted its growth up to 791% on day 14, and up to 300% after 28 days. In the case of biosurfactant produced by *L. pentosus,* at concentrations of 1 g/L, only was observed reduction growth for *S. aureus* and *A. brasiliensis* with maximum reduction percentages of 65.139% after 28 days and 87.727% after 7 days, respectively. However, for *E. coli*, *C. albicans* and *P. aeruginosa,* the effect is contrary, thus it promoted the growth of this strains up to 121%, 188% and 182% respectively.

The antimicrobial assay carried out in this work was based on the preservative Challenge testing, which helps to determine whether a substance will be effective in controlling microbial contamination in a cosmetic product during its shelf-life. Furthermore, it confirms its effectiveness in preventing the contamination that might be introduced during and after the manufacturing process of a cosmetic product.

In the literature, there are numerous examples of antimicrobial assays regarding biosurfactants. For instance, Das and collaborators [12] have achieved 100% inhibition of *E. coli* using the biosurfactant produced by *L. plantarum*; whereas Gudiña et al., [13,14] have obtained 50.9% and 13.5% of inhibition against *P. aeruginosa* using biosurfactants produced by *L. paracasei* and *Lactobacillus agilis* CCUG31450, respectively. Most of these results are focused on the growth of pathogenic microorganisms, analyzing the capacity of biosurfactant to inhibit their growth, in comparison with samples in absence of biosurfactants. However, the antimicrobial assay carried out in this work, is based on the capacity of two biosurfactant extracts to kill pathogenic microorganisms, already present in a specific media, at their optima conditions.

In comparison with other assays, Rivardo et al., [20] have observed a synergistic effect between a biosurfactant, obtained from *Bacillus licheniformis,* and various antibiotics against a mature 24 h uropathogenic *E. coli* CFT073 biofilm, corroborating that biosurfactants can be good preservatives agents. Although, they observed that the biosurfactant produced by *B. licheniformis* alone was not able to remove the adherent cells of the pre formed biofilm. The activity observed on the assay of *L. pentosus* showed a similar behavior, due to this biosurfactant extract was not able to eliminate the pathogenic microorganisms either.

The results obtained for biosurfactant extract from CSL are more in agreement with those achieved by Zouari and coworkers [54], which have demonstrated that a lipopeptide biosurfactant, produced by *Bacillus subtilis* SPB1, possess antimicrobial, anti-wrinkle, moisturizing and free radical scavenging properties, observing low cytotoxicity against human cells. Moreover, Lydon et al., [55] have observed that glycolipid biosurfactants, namely sophorolipids produced by the yeast *Starmerella bombicola*, showed important antimicrobial activity against the nosocomial infective agents *Enterococcus faecalis* and

*P. aeruginosa*, achieving significant reductions in CFU at concentrations of 5 g/L; although its antimicrobial activity was lower than that produced by the biosurfactant extract from CSL. In the case of *L. pentosus*, the antimicrobial activity observed was, in general, much lower than those reported in literature [55], probably caused by using five-fold concentrations.

It is important to notice that most of the biosurfactants studied in the literature showed good bacteriostatic properties (agent that stops bacteria from reproducing, while not necessarily killing them), but without achieving a bactericide activity (substance that kills bacteria). However, the biosurfactant extract obtained from corn milling industry, showed a strong response as preservative agent against *S. aureus*, *E. coli*, *P. aeruginosa* and *A. brasiliensis.* In addition, this biosurfactant extract has an important advantage, in comparison with other biosurfactants, due to it is obtained directly from an agroindustrial stream, with a reduced industrial production cost.

***Irritant response of the biosurfactant extracts on biological membranes***

The CAM of different hen´s eggs was observed over a period of 5 min, after the addition of solutions containing both biosurfactant extracts, SDS, NaOH (positive control) or PBS (negative control). SDS was included in the experiment, also as positive control, to compare the effects between a common chemical surfactant, amply used in cosmetic and pharmaceutical industry, and two novel biosurfactant extracts with potential uses in these kind of industries. As it can be observed in **Figure 2A**, SDS produced hemorrhage and vascular lysis in the CAM, detected by the presence of bleeding from the vessels and blood vessel disintegration. However, the addition of the biosurfactant extracts, studied in this work, at the same concentration, did not produce any negative effect in the CAM (**Figure 2B and 2C**).

In 1991 the German Federal Health Office coordinated a study to validate alternative methods to the Draize rabbit's eye test used to classify the substances included in cosmetic and pharmaceutical formulations, according with their irritant capacity on mucous membranes, observing a good correlation between this test and the results obtained with the HET-CAM assay [56]. Therefore, the potential irritability of a substance may be detected observing adverse changes, which occur in CAM of hen´s eggs after an exposure time. Taking into account the protocol established by ICCVAM [30], those substances at threshold concentrations between 1% and 2.5% with an Irritation Index (II) < 16 are classified as irritant. At the concentration used, both biosurfactant extracts did not produce any hemorrhage, coagulation and vessel lysis in the CAM of a hen´s egg, whereas SDS, at same concentration, gave an II of 6.55 as exhibited in **Table 5**. The classification obtained for SDS with this technique was comparable with the one published for sodium laureth sulphate surfactant, being both of them considered as moderate irritanting substances [57]. These authors defined this compound as moderately irritant, observing hemorrhage and lysis occurrences in the CAM of the hen's egg in its presence. The occurrence of vascular injury or coagulation in response to a compound is the basis for employing this technique, as an indication that a product or substance would damage mucous membranes, especially the eye, in assays carry out *in vivo* [58].

The use of biosurfactants could avoid the damage produced on skin by chemical surfactants, which could remove skin lipids and could cause corneocytes swelling, skin roughness and transepidermal water loss [49]. Additionally, the biosurfactant extracts could be used in the pharmaceutical industry to improve the poor solubility and low solution rate of poorly water-soluble drugs [8]. Usually, water is the solvent of choice for liquid pharmaceutical formulations and most of the drugs possess poor aqueous solubility [8].

## CONCLUSIONS

In the current work, two biosurfactants were compared in terms of antimicrobial activity and irritant capacity at the same concentration. It has been observed that a biosurfactant extract, obtained from corn milling industry, has important preservative capacities against *S. aureus, E. coli* and *P. aeruginosa,* killing all these germs in 7 to 14 days. Additionally, it has an inhibitory effect on *A. brasiliensis* reducing the growth of this microorganism up to 95%, after 14 days. However, the preservative capacity of the biosurfactant extract produced by *L. pentosus,* gave completely different results. In this case, the extract from *L. pentosus* at the concentration tested, improved the growth of pathogenic microorganisms, except in the case of *S. aureus* and *A. brasiliensis*, after 28 and 14 days, respectively. In general, this work proves the potential of the biosurfactant extract obtained from CSL as preservative against aerobic bacteria; whereas the *L. pentosus* extract has no preservative activity against aerobic bacteria or moulds at the concentration tested.

On the other hand, in terms of irritation on biological membranes, neither biosurfactant from CSL nor the one produced by *L. pentosus* produced any vascular or coagulation injury in the chorioallantoic membrane of the hen's egg. However, SDS used as control produced intravascular coagulation.

This work proves the potential of the biosurfactant extract obtained from CSL as preservative against bacteria and surface-active agent, in comparison with chemical surfactants, with potential applications in cosmetic industry, as they did not affect sensitive biological membranes.

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**REFERENCES**

1. Yazar K, Johnsson S, Lind M-L, Boman A, Lidén C. Preservatives and fragrances in selected consumer-available cosmetics and detergents. *Contact Dermatitis*. 2011;64(5):265-272. doi:10.1111/j.1600-0536.2010.01828.x

2. Ahmad V, Khan MS, Jamal QMS, Alzohairy MA, Al Karaawi MA, Siddiqui MU. Antimicrobial potential of bacteriocins: in therapy, agriculture and food preservation. *Int J Antimicrob Agents*. 2017;49(1):1-11. doi:10.1016/j.ijantimicag.2016.08.016

3. Mnif I, Ghribi D. Glycolipid biosurfactants: main properties and potential applications in agriculture and food industry. *J Sci Food Agric*. 2016;96(13):4310-4320. doi:10.1002/jsfa.7759

4. Sieniawska E, Świątek Ł, Rajtar B, Kozioł E, Polz-Dacewicz M, Skalicka-Woźniak K. Carrot seed essential oil—Source of carotol and cytotoxicity study. *Ind Crops Prod*. 2016;92:109-115. doi:10.1016/j.indcrop.2016.08.001

5. Taofiq O, González-Paramás AM, Martins A, Barreiro MF, Ferreira ICFR. Mushrooms extracts and compounds in cosmetics, cosmeceuticals and

nutricosmetics—A review. *Ind Crops Prod*. 2016;90:38-48. doi:10.1016/j.indcrop.2016.06.012

6. Vecino X, Cruz JM, Moldes AB, Rodrigues LR. Biosurfactants in cosmetic formulations: trends and challenges. *Crit Rev Biotechnol*. 2017;37(7):911-923. doi:10.1080/07388551.2016.1269053

7. Mehling A, Kleber M, Hensen H. Comparative studies on the ocular and dermal irritation potential of surfactants. *Food Chem Toxicol*. 2007;45(5):747-758. doi:10.1016/j.fct.2006.10.024

8. Savjani KT, Gajjar AK, Savjani JK. Drug Solubility: Importance and Enhancement Techniques. *ISRN Pharm*. 2012;2012:1-10. doi:10.5402/2012/195727

9. de Lucas-Gil E, Rubio-Marcos F, Leret P, et al. Opening a New Gate to Glass Preservative with Long-Lasting Antimicrobial Activity as Replacement of Parabens. *ACS Sustain Chem Eng*. 2017;5(1):294-302. doi:10.1021/acssuschemeng.6b01609

10. Csáki KF. Synthetic surfactant food additives can cause intestinal barrier dysfunction. *Med Hypotheses*. 2011;76(5):676-681. doi:10.1016/j.mehy.2011.01.030

11. De S, Malik S, Ghosh A, Saha R, Saha B. A review on natural surfactants. *RSC Adv*. 2015;5(81):65757-65767. doi:10.1039/C5RA11101C

12. Das P, Mukherjee S, Sen R. Antimicrobial potential of a lipopeptide biosurfactant derived from a marine Bacillus circulans. *J Appl Microbiol*.

2008;104(6):1675-1684. doi:10.1111/j.1365-2672.2007.03701.x

13. Gudiña EJ, Rocha V, Teixeira JA, Rodrigues LR. Antimicrobial and antiadhesive properties of a biosurfactant isolated from *Lactobacillus paracasei* ssp. *paracasei* A20. *Lett Appl Microbiol*. 2010;50(4):419-424. doi:10.1111/j.1472-765X.2010.02818.x

14. Gudiña EJ, Fernandes EC, Teixeira JA, Rodrigues LR. Antimicrobial and anti-adhesive activities of cell-bound biosurfactant from Lactobacillus agilis CCUG31450. *RSC Adv*. 2015;5(110):90960-90968. doi:10.1039/C5RA11659G

15. Madhu AN, Prapulla SG. Evaluation and functional characterization of a biosurfactant produced by Lactobacillus plantarum CFR 2194. *Appl Biochem Biotechnol*. 2014;172(4):1777-1789. doi:10.1007/s12010-013-0649-5

16. Rodrigues LR, Teixeira JA, van der Mei HC, Oliveira R. Physicochemical and functional characterization of a biosurfactant produced by Lactococcus lactis 53. *Colloids Surfaces B Biointerfaces*. 2006;49(1):79-86. doi:10.1016/j.colsurfb.2006.03.003

17. Rodrigues LR, Teixeira JA, van der Mei HC, Oliveira R. Isolation and partial characterization of a biosurfactant produced by Streptococcus thermophilus A. *Colloids Surfaces B Biointerfaces*. 2006;53(1):105-112. doi:10.1016/j.colsurfb.2006.08.009

18. Hassan M, Essam T, Yassin AS, Salama A. Optimization of rhamnolipid production by biodegrading bacterial isolates using Plackett–Burman design. *Int J Biol Macromol*. 2016;82:573-579. doi:10.1016/J.IJBIOMAC.2015.09.057

19. Chakraborty S, Ghosh M, Chakraborti S, et al. Biosurfactant produced from Actinomycetes nocardiopsis A17: Characterization and its biological evaluation. *Int J Biol Macromol*. 2015;79:405-412. doi:10.1016/j.ijbiomac.2015.04.068

20. Rivardo F, Martinotti MG, Turner RJ, Ceri H. Synergistic effect of lipopeptide biosurfactant with antibiotics against Escherichia coli CFT073 biofilm. *Int J Antimicrob Agents*. 2011;37(4):324-331. doi:10.1016/j.ijantimicag.2010.12.011

21. Vecino X, Rodríguez-López L, Ferreira D, Cruz JM, Moldes AB, Rodrigues LR. Bioactivity of glycolipopeptide cell-bound biosurfactants against skin pathogens. *Int J Biol Macromol*. 2018;109:971-979. doi:10.1016/j.ijbiomac.2017.11.088

22. López-Prieto A, Rodríguez-López L, Rincón-Fontán M, Moldes AB, Cruz JM. Effect of biosurfactant extract obtained from the corn-milling industry on probiotic bacteria in drinkable yogurt. *J Sci Food Agric*. 2018;(May). doi:10.1002/jsfa.9251

23. Vecino X, Barbosa-Pereira L, Devesa-Rey R, Cruz JM, Moldes AB. Optimization of liquid–liquid extraction of biosurfactants from corn steep liquor. *Bioprocess Biosyst Eng*. 2015;38(9):1629-1637. doi:10.1007/s00449-015-1404-9

24. Kim SH, Lim EJ, Lee SO, Lee JD, Lee TH. Purification and characterization of biosurfactants from Nocardia sp. L-417. 2000;31(3):249-253.

25. Marchant R, Banat IM. Biosurfactants: A sustainable replacement for chemical surfactants? *Biotechnol Lett*. 2012;34(9):1597-1605. doi:10.1007/s10529-012-0956-x

26. Anschau A, Caruso CS, Kuhn RC, Franco TT. Validation of the sulfo-

phosphovanillin (SPV) method for the determination of lipid content in oleaginous microorganisms. *Brazilian J Chem Eng*. 2017;34(1):19-27. doi:10.1590/0104-6632.20170341s20140222

27. Rodríguez-López L, Vecino X, Barbosa-Pereira L, Moldes AB, Cruz JM. A multifunctional extract from corn steep liquor: antioxidant and surfactant activities. *Food Funct*. 2016;7(9):3724-3732. doi:10.1039/C6FO00979D

28. Mariotti F, Tomé D, Mirand PP. Converting nitrogen into protein - Beyond 6.25 and Jones' factors. *Crit Rev Food Sci Nutr*. 2008;48(2):177-184. doi:10.1080/10408390701279749

29. Committee CTN 84. Cosmetics-Microbiology-Evaluation of the antimicrobial protection of a cosmetic product. UNE-EN-ISO-11930:2012. 2014.

30. National Toxicology Program. ICCVAM-Recommended Test Method Protocol: Hen's Egg Test – Chorioallantoic Membrane (HET-CAM) Test Method. 2010. http://iccvam.niehs.nih.gov/methods/ocutox/MildMod-TMER.htm. Accessed May 8, 2018.

31. Rincón-Fontán M, Rodríguez-López L, Vecino X, Cruz JM, Moldes AB. Design and characterization of greener sunscreen formulations based on mica powder and a biosurfactant extract. *Powder Technol*. 2018;327:442-448. doi:10.1016/j.powtec.2017.12.093

32. Rincón-Fontán M, Rodríguez-López L, Vecino X, Cruz JM, Moldes AB. Influence of micelle formation on the adsorption capacity of a biosurfactant extracted from corn on dyed hair. *RSC Adv*. 2017;7(27):16444-16452. doi:10.1039/C7RA01351E

33. Patil HI, Pratap AP. Production and Quantitative Analysis of Trehalose Lipid Biosurfactants Using High-Performance Liquid Chromatography. *J Surfactants Deterg*. 2018;21(4):553-564. doi:10.1002/jsde.12158

34. de Faria AF, Teodoro-Martinez DS, de Oliveira Barbosa GN, et al. Production and structural characterization of surfactin (C14/Leu7) produced by Bacillus subtilis isolate LSFM-05 grown on raw glycerol from the biodiesel industry. *Process Biochem*. 2011;46(10):1951-1957. doi:10.1016/J.PROCBIO.2011.07.001

35. Wan C, Chen S, Wen L, Liu X, Lee D-J, Yang X. Biosynthesis, characterization and potentiality of lipopeptides produced by Bacillus flexus S1 without inductive carbon sources. *RSC Adv*. 2016;6(88):85074-85082. doi:10.1039/C6RA17510D

36. Ohadi M, Dehghannoudeh G, Forootanfar H, Shakibaie M, Rajaee M. Investigation of the structural, physicochemical properties, and aggregation behavior of lipopeptide biosurfactant produced by Acinetobacter junii B6. *Int J Biol Macromol*. 2018;112:712-719. doi:10.1016/j.ijbiomac.2018.01.209

37. Vecino X, Barbosa-Pereira L, Devesa-Rey R, Cruz JM, Moldes AB. Optimization of extraction conditions and fatty acid characterization of *Lactobacillus pentosus* cell-bound biosurfactant/bioemulsifier. *J Sci Food Agric*. 2015;95(2):313-320. doi:10.1002/jsfa.6720

38. Vecino X, Barbosa-Pereira L, Devesa-Rey R, Cruz JM, Moldes AB. Study of the surfactant properties of aqueous stream from the corn milling industry. *J Agric Food Chem*. 2014;62(24):5451-5457. doi:10.1021/jf501386h

39. Ramirez EC, Johnston DB, McAloon AJ, Yee W, Singh V. Engineering process

and cost model for a conventional corn wet milling facility. *Ind Crops Prod*. 2008;27(1):91-97. doi:10.1016/j.indcrop.2007.08.002

40. Murthy HN, Lee E-J, Paek K-Y. Production of secondary metabolites from cell and organ cultures: strategies and approaches for biomass improvement and metabolite accumulation. *Plant Cell, Tissue Organ Cult*. 2014;118(1):1-16. doi:10.1007/s11240-014-0467-7

41. Swak JG, Herbert KL. Optical Damage and Recovery of the in Vitro Bovine Ocular Lens for Alcohols, Surfactants, Acetates, Ketones, Aromatics, and Some Consumer Products: A Review. *J Toxicol Cutan Ocul Toxicol*. 1997;16(3):173-187. doi:10.3109/15569529709048894

42. Sa R-B, An X, Sui J-K, et al. Purification and structural characterization of fengycin homologues produced by Bacillus subtilis from poplar wood bark. *Australas Plant Pathol*. 2018;47(3):259-268. doi:10.1007/s13313-018-0552-1

43. Bie X, Lu Z, Lu F. Identification of fengycin homologues from Bacillus subtilis with ESI-MS/CID. *J Microbiol Methods*. 2009;79(3):272-278. doi:10.1016/j.mimet.2009.09.013

44. Li J, Deng M, Wang Y, Chen W. Production and characteristics of biosurfactant produced by Bacillus pseudomycoides BS6 utilizing soybean oil waste. *Int Biodeterior Biodegradation*. 2016;112:72-79. doi:10.1016/j.ibiod.2016.05.002

45. Sabturani N, Latif J, Radiman S, Hamzah A. *Spectroscopic Analysis of Rhamnolipid Produced by Pseudomona Aeruginosa UKMP14T (Analisis Spektroskopik Ramnolipid Yang Dihasilkan Oleh P. Aeruginosa UKMP14T)*. Vol 20.; 2016. http://www.ukm.my/mjas/v20_n1/pdf/Noramiza_20_1_4.pdf.

Accessed October 10, 2018.

46. Gupta R, Prabhune AA. Structural determination and chemical esterification of the sophorolipids produced by Candida bombicola grown on glucose and α-linolenic acid. *Biotechnol Lett*. 2012;34(4):701-707. doi:10.1007/s10529-011-0818-y

47. Campos JM, Stamford TLM, Sarubbo LA. Production of a Bioemulsifier with Potential Application in the Food Industry. *Appl Biochem Biotechnol*. 2014;172(6):3234-3252. doi:10.1007/s12010-014-0761-1

48. Gupta S, Raghuwanshi N, Varshney R, et al. Accelerated in vivo wound healing evaluation of microbial glycolipid containing ointment as a transdermal substitute. *Biomed Pharmacother*. 2017;94:1186-1196. doi:10.1016/j.biopha.2017.08.010

49. Förster T, Issberner U, Hensen H. Lipid/surfactant compounds as a new tool to optimize skin-care properties of personal-cleansing products. *J Surfactants Deterg*. 2000;3(3):345-352. doi:10.1007/s11743-000-0138-8

50. van Smeden J, Bouwstra JA. Stratum Corneum Lipids: Their Role for the Skin Barrier Function in Healthy Subjects and Atopic Dermatitis Patients. In: ; :8-26. doi:10.1159/000441540

51. Drake DR, Brogden KA, Dawson D V., Wertz PW. Thematic Review Series: Skin Lipids . Antimicrobial lipids at the skin surface. *J Lipid Res*. 2008;49(1):4-11. doi:10.1194/jlr.R700016-JLR200

52. Secchi G. Role of protein in cosmetics. *Clin Dermatol*. 2008;26(4):321-325.

doi:10.1016/J.CLINDERMATOL.2008.04.004

53. Ferreira A, Vecino X, Ferreira D, Cruz JM, Moldes AB, Rodrigues LR. Novel cosmetic formulations containing a biosurfactant from Lactobacillus paracasei. *Colloids Surfaces B Biointerfaces*. 2017;155:522-529. doi:10.1016/j.colsurfb.2017.04.026

54. Zouari R, Moalla-Rekik D, Sahnoun Z, Rebai T, Ellouze-Chaabouni S, Ghribi-Aydi D. Evaluation of dermal wound healing and in vitro antioxidant efficiency of Bacillus subtilis SPB1 biosurfactant. *Biomed Pharmacother*. 2016;84:878-891. doi:10.1016/j.biopha.2016.09.084

55. Lydon HL, Baccile N, Callaghan B, Marchant R, Mitchell CA, Banat IM. Adjuvant Antibiotic Activity of Acidic Sophorolipids with Potential for Facilitating Wound Healing. *Antimicrob Agents Chemother*. 2017;61(5):e02547-16. doi:10.1128/AAC.02547-16

56. Spielmann H, Gerner I, Kalweit S, et al. Interlaboratory assessment of alternatives to the Draize eye irritation test in Germany. *Toxicol Vitr*. 1991;5(5-6):539-542. doi:10.1016/0887-2333(91)90089-V

57. Steiling W, Bracher M, Courtellemont P, De Silva O. The HET-CAM, a useful in vitro assay for assessing the eye irritation properties of cosmetic formulations and ingredients. *Toxicol Vitr*. 1999;13(2):375-384. doi:10.1016/S0887-2333(98)00091-5

58. Tavaszi J, Budai P. The use of HET-CAM test in detecting the ocular irritation. 2007;72(2):137-141.

## FIGURE CAPTIONS

**Figure 1:** ESI/MS spectra recorded for biosurfactant extract from CSL (A) and biosurfactant produced by *L. pentosus* (B).

**Figure 2.** Picture of HET-CAM in presence of SDS (A), biosurfactant extract from corn (B), or biosurfactant extract produced by *L. pentosus* (C), respectively.